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Exosomes of Malignant Tumors: Prospects of Omics Diagnostics

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Abstract—Early detection of the disease, prediction of its course, and response to therapy are the main problems in the diagnostics and treatment of malignant tumors. They may be solved by means of identification of biomarkers secreted by tumor cells within extracellular vesicles, particularly, exosomes. The study of exosomal proteins attracts special attention, because exosomal proteins can contain information about tumor identity, and also represent a set of signaling molecules, regulating processes of tumor progression and growth. In addition, analysis of exosomes secreted into the extracellular space corresponds to the promising concept of a liquid biopsy. In this review, we have summarized the current information about molecular studies of exosomes from various types of malignant tumors, including colorectal cancer, lung cancer, ovary, prostate and breast cancers, with special emphasis on omics methods and have considered prospects for their use in diagnostics.

Keywords: exosomes, omics diagnostics, proteomics, transcriptomics, malignant tumors

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INTRODUCTION

Exosomes are detected in almost all human biological fluids: blood, urine, saliva, amniotic and cerebrospinal fluids, bile, tears, breast milk, and seminal fluid. Although functions of exosomes are not fully understood, results of recent studies indicate their role in intercellular interactions and the regulation of the functions of the immune system. Moreover, exosomes play an important role in the biology of tumors, and are involved in the processes of malignancy, tumor growth, development of drug resistance of tumor cells, angiogenesis, and metastasis [1–3]. Almost all cell types can produce exosomes containing specific proteins, lipids, RNA, and DNA [4]. Since the exosome composition depends on the cell origin and reflects physiological and pathological state of the cell, analysis of the molecular cargo of exosomes represents an attractive approach for the search for new diagnostic, prognostic, and predictive markers.

Abbreviations used: CEA—cancer embryonic antigen; CRC—colorectal cancer; EGFR—epidermal growth factor receptor; ELISA—enzyme-linked immunosorbent assay; ESCRT—endosomal sorting complex; HPLC-MS/MS—high performance liquid chromatography coupled with tandem mass spectrometry; MRM—multiple reaction monitoring; MVs—microvesicles; NSCLC—non-small cell lung cancer; nSMase—sphingomyelinase; PCa—prostate cancer; PEG—polyethylene glycol; PSA—Prostate Specific Antigen; SILAC—Stable Isotope Labeling with Amino acids in Cell culture.

1. HISTORY OF STUDY, BIOGENESIS, CHARACTERISTIC MOLECULAR COMPOSITION OF EXOSOMES

The exosome research is a relatively young direction in biological science. The term "exosomes" was originally applied to membrane vesicles obtained from biological fluids during analysis of reticulocyte differentiation [5, 6]. Initially, the term exosomes was used to define vesicles of 40–100 nm in size, secreted exclusively by reticulocytes during differentiation; later it was found that B-lymphocytes and dendritic cells were also able to secrete exosomes [7, 8]. In addition, it was demonstrated that hematopoietic cells, cytotoxic T-lymphocytes, platelets, neuronal and mast cells also released exosomes by fusion of intracellular multivesicular endosomes with the plasma membrane [9, 10].

In the first studies, the process of exosome release from reticulocytes was considered as one of the mechanisms for "waste" withdrawal from cells [11]. Then it has become increasingly clear that exosomes are involved in intercellular interactions and in a complex regulatory system of the immune system functioning. In 2013, Randy Schekman, James Rothman, and Thomas Südhof were awarded with the Nobel Prize in Physiology and Medicine for the discovery of vesicular transport, the main transport system of the cell. Further studies and understanding of the role and functions of exosomes attracted attention of researchers around the world and it was found that the vesicles could play an important role in many physiological and pathological processes.

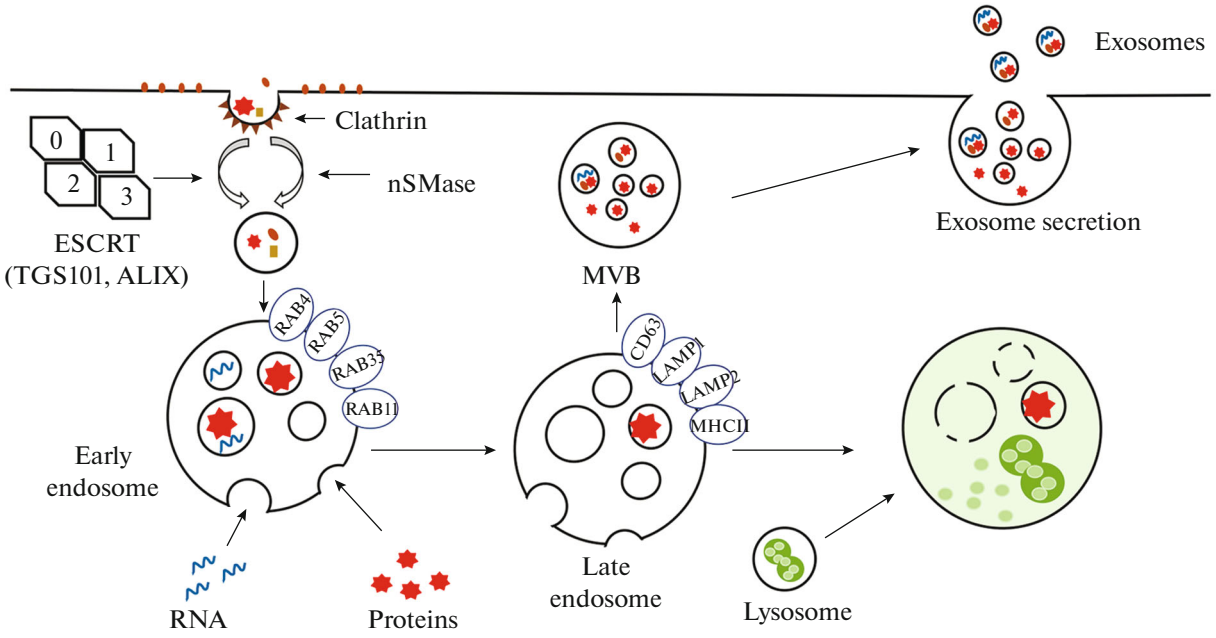

Fig. 1. The scheme of exosome biogenesis. 0, 1, 2, 3 ESCRT—components of the endosomal sorting complex, nSMase—sphingomyelinase, MVB—multivesicular body. Explanations are given in the text.

Various types of mammalian cells secrete exosomes into the extracellular medium by fusion of intracellular multivesicles (MVs) with the cell membrane [5, 12]. The characteristic feature of exosome formation, which differs from direct budding of the plasma membrane, includes the fusion of multivesicular bodies (endosomes in essence) with the plasma membrane as opposed to direct budding of the plasma membrane. The exosome formation can proceed either via the classical pathway involving ESCRT, or via the ESCRT-independent mechanism (Fig. 1).

The process of exosome formation via the classical pathway begins with formation of clathrin-coated membrane invaginations. Then, invaginated vacuoles develop into early endosomes loaded with ubiquitinylated products: this process needs involvement of various components of the ESCRT sorting complex (ESCRT-0, -1, -2, -3). The next step is the secondary invagination of the walls of the early exosomes, which leads to the formation of intraluminal vesicles that accumulate and mature within the early endosomes. Subsequent acidification of the internal content of endosomes due to the transfer of protons by the V-ATPase leads to their maturation in late endosomes [13]. The late endosomes with intraluminal vesicles are denominated as multivesicular bodies. Subsequently, multivesicular bodies either remain in the cytoplasm and, fusing with lysosomes, participate in protein degradation, or fusing with the plasma membrane are secreted into the extracellular space in the form of exosomes [14].

The exosome formation via the ESCRT-independent mechanism involves nSMase at the stage of formation of invaginated vacuoles (this enzyme cleaves sphingomyelin to ceramide); in addition, tetraspanins CD63, CD9, and CD81 play an important role in exosome biogenesis [15]. The detailed mechanism of exosome formation via the ESCRT-independent mechanism is not fully understood.

The membrane structure of exosomes provides protection of the internal components, and therefore exosomal transport represents an effective way of influencing not only surrounding cells, but also on more distant target cells. Cell-secreted exosomes can transport to target cells many biological components, including mRNA, miRNA, DNA fragments, lipids, and proteins [16, 17].

The composition of a specific exosome proteome includes proteins associated only with the type of exosome-secreting cells, as well as proteins found in most exosomes, regardless of the source of origin. In general, proteins of the plasma membrane, endosomes, and cytosol are more common in exosomes, while proteins of the nucleus, mitochondria, endoplasmic reticulum, and Golgi apparatus are represented to a lesser extent.

Among the membrane proteins found in exosomes, tetraspanins CD9, CD37, CD53, CD63, CD81, and CD82 are widely represented [18]. The proteins CD9, CD63, and CD81 are most frequently used for immunoblotting to confirm the effectiveness of exosome isolation.

A number of proteins that are most universal for exosomes are involved in their biogenesis and are characteristic of endosomes. Proteins such as TSG101, clathrin, and ALIX/PDCD6IP (programmed cell death 6-interacting protein) are associated with the ESCRT sorting complex [19]. A number of exosome proteins are markers of their origin from late (CD63, LAMP1 and LAMP2 proteins associated with lysosomes, histocompatibility complex class II and RAB7) or early (RAB4, RAB5, RAB11 and RAB35 proteins) endosomes [15].

Vesicles isolated from various body fluids contain lipid raft binding proteins, such as flotillins and GPI-anchored proteins [20].

Among various types of RNA, microRNA is particularly interesting; it is transported by exosomes and can influence gene expression in distant cells.

In the context of lipid composition, exosomes are enriched with cholesterol, phosphatidylserine and sphingomyelin in comparison with the cell membrane.

Search for exosome markers requires clear understanding of experimental conditions used in such studies as many factors, including pro-inflammatory signals, hypoxia, oncogen expression, and low pH values of the cultivation medium have a significant impact on qualitative composition of exosome molecules [15]. In addition, the qualitative determination of exosome markers that convincingly distinguish them from other extracellular vesicles is complicated by the heterogeneous nature of biogenesis itself and the low specificity of exosome isolation methods, such as ultracentrifugation and precipitation. In this context, quantitative determination of marker proteins may become promising: for example, in our laboratory a method was developed for quantitative determination of the marker proteins CD63, CD82, and HSPA8 in plasma exosomes by means of quantitative targeted mass spectrometry [21].

1.1. In silico Sources for Searching for Exosome Marker Proteins

The potential use of the molecular cargo of exosomes as diagnostic markers needs a careful analysis of these particles and the presentation of the results in a format convenient for the scientific community. Considering importance of already obtained data and growing interest in this field in the future, the open web resource ExoCarta (http://exocarta.ludwig.edu.au) was created in 2009. This portal is a database that has cataloged the molecules identified in exosomes in recent years; it also contains information on exosome isolation methods, purification procedures, used samples, and information about the researcher [22].

Among 100 molecules most often found in exosomes (regardless of the source of their origin), there are proteins involved in signal transmission, such as tetraspanins (CD9, CD63, CD81), integrins (ITB1, ITA6), nucleotide exchange factors, and GTP-binding proteins (GNAI2, GNAS1, GNAS2, and ARF1), adapter proteins (proteins 14-3-3 alpha/beta, gamma, zeta/delta, eta and theta) and annexins 2, 5, 6 and 11. However, it should be noted that among these 100 molecules of the ExoCarta database there are many non-specific proteins (e.g. actin, alpha-2-macroglobulin or serum albumin), which are possibly artefacts of exosome isolation from biological fluids and cell culture media.

The material from the ExoCarta database can be useful in planning a targeted study of the molecular composition of exosomes, for example, for development of the MRM method for analysis of proteins.

The ExoCarta database is part of the Vesiclepedia resource, which summarizes current knowledge and experimental data for various types of extracellular vesicles. In addition to ExoCarta, information on the molecular composition of various extracellular vesicles, including exosomes, is available at the large EVpedia database (http://evpedia.info).

2. THE ROLE OF EXOSOMES IN TUMOR BIOLOGY AS A METASTASIS MEDIATOR

Exosomes are part of a widespread transport network through which cells exchange information with each other via transfer of DNA, RNA, proteins, and membrane-bound factors. In this context, exosomes secreted by tumor cells directly affect the tumor microenvironment, and, due to their spread through the blood and lymph, affect distant sites, thus creating a pre-metastatic niche that promotes metastasis, which Tung and colleagues denominated as the tumor macroenvironment [23]. It is believed that the main mechanism of the exosome action consists in immunosuppression. The signal of immunological tolerance mediated by tumor-derived exosomes leads to inhibition of proliferation of immune cells, impaired differentiation of monocytes, as well as apoptosis of activated $CD8^+$ T-lymphocytes and stimulation of regulatory T-cells that induce paracrine immune suppression [24].

Exosomes also contribute to metastasis due to metabolic reprogramming, which results in the transition of cells from the use of oxidative phosphorylation as the main energy source to glycolysis and increases acidification of the extracellular matrix, which promotes tumor growth [25].

Moreover, exosomes are able to activate oncogenes in the cells of the target organ for metastasis due to the transfer of oncogenic proteins from the primary tumor. For example, it was shown that the epidermal growth factor receptor (EGFR) from gastric cells was transported by cancer exosomes to stromal cells of the liver and triggered expression of hepatocyte oncogenic growth factor in them [26]. In the case of colorectal

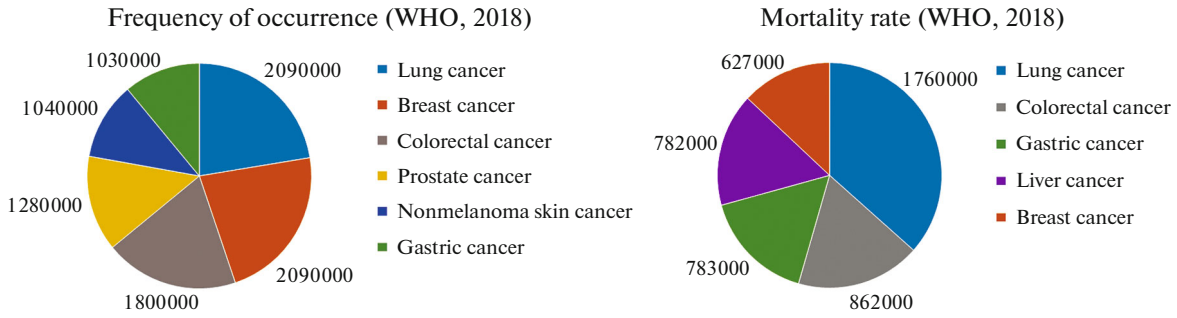

Fig. 2. The prevalence and frequency of deaths for the main types of malignant tumors. Adapted from [28].

cancer (CRC), in which mutations in the *KRAS* gene are frequent, it has been shown that tumor cell exosomes transfer oncogenic proteins, the mutant form of KRAS, EGFR, and integrins – into tumor cells carrying the wild-type *KRAS* gene, contributing to metastasis and disease progression [27].

Taking into consideration a powerful and versatile contribution of exosomes to metastasis, their molecular composition represents a rich source of prognostic markers that reflect the degree of aggressiveness of the malignant tumor.

3. APPLICATION OF OMICS APPROACHES FOR SEARCHING BIOLOGICAL MARKERS OF MALIGNANT TUMORS IN EXOSOMES

According to WHO, oncological diseases take the second place in the list of the main causes of death after diseases of the cardiovascular system. In 2018, 9.6 million people died from malignant tumors. At the same time, lung cancer, breast cancer, and colorectal cancer are leading in terms of prevalence, while lung cancer, colorectal cancer, and gastric cancer are among the top three in the frequency of deaths (Fig. 2); they will be discussed below.

It is well known that the difference between the mortality rates of patients with early and late diagnosed stages of cancer is very significant. Modern methods of therapy significantly prolong the life of patients with the initial forms of many oncological diseases; this means that reducing mortality from cancer can be achieved by introducing methods of early diagnosis. Moreover, using predictive and prognostic markers it is possible to predict the response to the treatment and the severity of the disease in various patients, thus providing an individual approach to the patient, which meets the goals of personalized medicine.

Currently, the search for tumor markers, applicable for creation of new methods of laboratory diagnostics, is one of the priorities of fundamental oncology. Over the past few years, the prospects of developing methods for the cancer diagnostics based on the analysis of circulating exosomes as factors involved in local tumor growth and in the generalization of the tumor process have been proved [29]. The main advantage of this approach is the non-invasive nature of the study, which avoids the risks associated with biopsies and partially solves the problem of tumor heterogeneity.

The rapid development of genomic and proteomic methods has provided powerful technological platforms for the scientific community; these include genome-wide sequencing, genome-wide transcriptome analysis, shotgun and targeted mass spectrometric analysis. Such methods are characterized by high sensitivity, accuracy, specificity, and high performance. Table 1 shows that in the context of cancer, the exosome researchers actively employed the new opportunities provided by omics approaches.

Below we consider in more details the examples of studies on tumor exosomes that prove the prospects of using these microvesicles as a source of biomarkers.

3.1. Lung Cancer

Lung cancer is the leading cause of cancer death (WHO official data [28]). The high mortality rate is partly explained by the fact that, according to the American Association of Pulmonologists, only 16% of patients are diagnosed with a localized tumor at an early stage (https://www.lung.org/lung-health-and-diseases/lung-disease-lookup/lung-cancer/resource-library/lung-cancer-fact-sheet.html). In this regard, the development of screening methods is an important task in the diagnosis of lung cancer. The study of the molecular composition of exosomes secreted by lung cancer cells is of particular interest to the scientific community from the diagnostic viewpoint [30, 31].

One of the omics directions is analysis of microRNAs, the class of small non-coding RNAs with abnormal expression in several types of cancer, which indicates their key role in the pathogenesis of these diseases [32]. MicroRNAs bind to the 3'-untranslated region (3'-UTR) of the target mRNA and this results in altered expression of the corresponding gene. Although microRNA profiling has shown promising results, its use is currently limited by tissue biopsy.

Table 1. The results of studies of the molecular load of exosomes in various types of tumors using omics approaches

The source of exosomes	Molecules identified in exosomes		Reference
Lung cancer			
Plasma	microRNA	hsa-miR-17-3p, hsa-miR-21, hsa-miR-106a, hsa-miR-146, hsa-miR-155, hsa-miR-191, hsa-miR-192, hsa-miR-203, hsa-miR-205, hsa-miR-210, hsa-miR-212 and hsa-miR-214	[32]
Biopsy	–	Increased content of EGFR	[37]
Plasma	Proteins	NY-ESO-1, EGFR, PLAP, EpCam, Alix	[38]
Cell lines A549, HCC827, HBE	Proteins of the ESCRT complex	STAM1, STAM2, VPS37B, VPS37C, VPS4B, VTA1	[39]
	Proteins associated with exosome biogenesis	ALIX, TSG101, SDCBP, SDC1	
	Proteins involved on vesicular transport	VAMP2, VAMP8, SNX2, STX3	
	Tetraspanins	CD9, CD63, CD81	
Pleural effusion	Proteins associated with signal transduction, immunity, structural organization of cells and transport	ABI-1, BSG, CAV1, EGFR, GRB2, RAS and SRC protein families	[40]
Serum	Proteins	AHSG, ECM1	[41]
Colorectal cancer			
Cell line LIM1215	Tissue specific proteins	CEP55, EPHA2, KRT18, MUC13, MAPK4, MINK1, PCNA, CEA, EpCAM, EGFR, POLD1, PPP2R1B and RUVBL1	[44]
Cell line LIM1863	Proteins involved in proliferation and invasion of cancer cells or in signaling pathways associated with carcinogenesis	HGFR, AREG, EFNB1, EFNB2, EPHA2-8, EPHB1-4, CTNNB1, TNIK, CRK, GRB2, CEACAM1 and CEACAM5	[45]
Cell line SW480	Proteins associated with cell adhesion	ILK, ITGA1, ICAM1, CTNNB1, PKP2	[46]
Cell line SW620	Proteins associated with cancer progression and appearance of drug resistance	AARS, CCDC50, PAK4, ANXA1, LCK, LCP1, AK2, HSPA9	
Breast cancer			
Cell line MCF10A, MCF7 and MDA-MB-231	microRNA	miR-21, miR-1246	[47]
Plasma	microRNA	miR-21, miR-1246	
Cell line MDA-MB-231	Proteins associated with exosome biogenesis and cell cycle regulation	ACTB/G, TBA1C, K1C9, TPM4, FSCN1, TAGL2, PDC6I	[49]
	Proteins associated with regulation of apoptosis	TERA, ANXA5, HSP71, PRDX2, UBIQ, PDC6I	
	Proteins associated with focal adhesion	LAMC1, ACTB/G	
	Proteins associated with signaling pathways and modulation of the immune system during tumor progression	LG3BP, LAMC1, GBB2, ITA3, ITA6, B2MG	
Prostate cancer			
Semen fluid	Proteins	ACTB, GAPDH, PHGDH, LGALS3BP and SEMG1, SEMG1, SEMG2, PIP, FN1, ACPP, KLK3	[51]
Plasma	Proteins associated with carcinogenesis and tumor growth	DNA2, PIF1, FHL3, GSTO2, MELK, IRX5, MCM5, MTUS1	[52]
Ovarian cancer			
Cell line SKOV3, CAOV3	Proteins associated with angiogenesis	EBNA1, MTA1	[53]
Cell line OVCAR-3 and IGROV1	Proteins associated with carcinogenesis and metastasis	EpCAM, PCNA, TUBB, EGFR, APOE, CLDN3	[54]
Biopsy-derived cell lines	microRNA	let-7i, miR-16, miR-21 and miR-214	[55]

Since tumor-specific miRNAs associated with disease prognosis, including therapeutic resistance, have been identified, their presence in tumor exosomes can also be used as a prognostic indicator.

The Rabinowits's research team isolated exosomes from the blood of patients with lung adenocarcinoma and healthy donors by using a combination of size exclusion chromatography and immunoaffinity isolation of exosomes by EpCAM [33]. Researchers determined elevated levels for 12 specific exosomal microRNAs (miR-17-3p, miR-21, miR-106a, miR-146, miR-155, miR-191, miR-192, miR-203, miR-205, miR -210, miR-212, and miR-214) in patients with pulmonary adenocarcinoma as compared with the healthy control. Thus, a molecular signature was obtained, which the researchers compared with the stage of the disease [33]. In addition, the results showed that the content of exosomal miRNAs and miRNAs obtained from tumor cells did not have significant differences; consequently, the exosome RNA profile represents a "mirror reflection" of the molecular composition of the tumor.

Other studies have linked a low level of exosomal miR-146a-5p with resistance to cisplatin [34], and a high level of expression of exosomal miR-1246 and miR-208-a with resistance to radiotherapy in patients with lung adenocarcinoma [35].

Profiling of different miRNA sets in medium-sized cohorts of patients ($n = 40–60$) with lung adenocarcinoma and squamous cell carcinoma of the lung in comparison with the healthy control has shown rather high sensitivity (80–97%) and specificity (60–90%); this suggests the prospects of microRNA analysis for the diagnostics and screening of this disease [36, 37].

At the protein level, exosomal markers for lung cancer are mainly examined using antibody-based techniques (immunoblotting, ELISA). Using these approaches an increased content of EGFR in exosomes was demonstrated in lung cancer patients as compared with patients with chronic inflammatory lung diseases [38], and increased expression of membrane-bound protein NY-ESO-1 was determined as an unfavorable prognostic marker [39].

To date, relatively few results of studies performed using high-performance proteomic methods have been published on the protein content of exosomes secreted by lung cancer cells.

One of the most important studies was performed by Clark et al. [40]. The researchers used the normal bronchial tissue cell line and two non-small cell lung cancer cell lines (NSCLC) A549 and HCC827 as model objects. The exosome isolation from the cell cultures was carried out using a combination of PEG precipitation methods and subsequent the iodixanol (OptiPrep™) gradient ultracentrifugation under the control of electron microscopy and immunoblotting for determination of the exosomal markers, CD63 and ALIX. Using mass spectrometric profiling with preliminary SILAC labeling of cell culture proteins, 721 exosomal proteins were quantitatively evaluated [40]. In comparison with normal bronchial cells, the NSCLC-derived exosomes were enriched with extracellular matrix proteins (EDIL3, MFGE8, and HSPG2), proteases (PAPPA, CTSA and GGT3P), cell adhesion proteins (DSG2, CD151, CNTN1, EPCAM, MPZL1, MPZL2), membrane receptors (EGFR, GPRC5A, ITGB1, ITGB6 and TACSTD2), proteins involved in proliferation (GRB2, RALA) and enzyme modulator proteins (GNB1, TOM1L1). The researchers also found that after addition of tumor-derived exosome (from the A549 or HCC827 NSCLC cell lines) to the HBE3 immortalized bronchial cell line, cell proliferation significantly increased, thus indicating the role of exosomal proteins in induction of proliferation in normal epithelial cells. Therefore, the detected differentially expressed proteins can be considered as a panel of markers for the risk of metastasis [40].

Park et al. performed a proteomic study of pleural effusion microvesicles (MVs) of 3 patients with NSCLC [41]. After differential ultracentrifugation in the sucrose gradient and iodixanol density gradient (OptiPrep™), the resultant MVs were evaluated by electron microscopy and immunoblotting analysis of the markers CD63, ezrin, and beta-actin. Proteomic analysis was performed by HPLC-MS/MS with preliminary fractionation of proteins in a 1D-polyacrylamide gel. In total, researchers identified 912 exosomal proteins. Moreover, the number of proteins in the proteomic profile of exosomes associated with molecular transport, immune system functions, and cell motility was much higher as compared to the parent cells from which these exosomes originated. Subsequent comparison with literature data, revealed that 153 proteins identified in MVs were found to be characteristic of pleural effusion, while 67 proteins were associated with lung tissue. These included such diagnostically significant proteins as ABI-1, basidgin (BSG), caveolin 1 (CAV1), EGFR, GRB2, proteins of the RAS and SRC family, CEACAM5 and cytoplasmic inorganic pyrophosphatase (Ppase), specific for pleural effusion, as well as claudine 1, annexin 1, aquaporin 1, CEACAM6, specific for lung tissue. This set of proteins is potentially important for detecting lung cancer, but it is necessary to validate markers in the whole plasma or in exosomes isolated from plasma.

More recently, a group of Chinese scientists successfully validated alpha-2HS-glycoprotein (AHSG) and ECM1 markers, selected from mass spectrometric analysis of exosomes isolated from serum, in a cohort of 125 patients with lung cancer at various stages and 46 healthy donors [42]. Data analysis from patients with lung cancer at an early stage and healthy donors performed using a combination of two new biomarkers AHSG, ECM1 and the cancer-embryonic antigen (CEA) used in the clinical practice achieved the sensi-

tivity and specificity of the test of 85.7% and 84.4%, respectively [42].

Thus, high throughput proteomic methods and methods of microRNA analysis have a high potential for developing approaches to the screening and diagnostics of lung cancer.

3.2. Colorectal Cancer

CRC is the third most commonly diagnosed cancer and the second leading cause of cancer-related mortality. Employment of screening programs and new approaches to therapy over the past twenty years resulted in a decrease in the mortality rate by 35% [43]. The main problem consists in the resistance to therapy due to the tumor heterogeneity determined by mutations in various genes (e.g, such as *KRAS*, *NRAS*, *BRAF*, *PIK3CA*), as well as a poor prognosis for patients with metastatic CRC. Thus, the search for new prognostic and predictive markers, including those secreted by exosomes is an important problem.

According to the latest data, exosome formation by CRC cells is necessary not only for intercellular communication, but also for removal of tumor suppressor microRNAs (miR-193a) from cells: this stimulates tumor proliferation and growth [44].

In order to determine CRC specific exosomal proteins, the LIM1215 cell MVs were isolated by immunoaffinity separation using the surface antigen A33. The efficiency of exosome isolation was evaluated by immunoblotting using the markers A33, TSG101, and HSP70. Based on the results of high-resolution mass spectrometry and subsequent bioinformatics analysis, tissue-specific proteins in the exosomes of CRC cells were determined; these included antigen A33, cadherin 17, claudins 1, 3, and 7, centrosomal protein 55 kDa (CEP55), ephrin B1 and ephrin B2, ephrin type-A receptor 2 (EPHA2), keratin 18 (KRT18), mucin 13 (MUC13), protein kinases MAPK4 and MINK1, proliferating cell nuclear antigen (PCNA), CEA, EpCAM, EGFR, POLD1, PPP2R1B and RUVBL1 proteins [45]. This wide list includes promising diagnostically significant molecules, which require further validation.

Tauro et al. isolated exosomes from colorectal carcinoma cell culture LIM1863 to compare morphological and proteomic profiles of exosomes isolated by three different methods: ultracentrifugation, OptiPrep™ gradient centrifugation and using EpCAM antibodies coated magnetic beads [46]. Among 171 unique proteins identified in the study, there were proteins involved in the proliferation and invasion of cancer cells as proto-oncogenes, or components of the signaling pathways associated with carcinogenesis: HGFR, AREG, EFNB1, EFNB2, EPHA2-8, EPHB1-4, CTNNB1, TNIK, CRK, GRB2, CEACAM1, and CEACAM5.

Researchers from Korea performed a comparative proteomic analysis of exosomes obtained from primary human CRC cells (SW480) and their metastatic derivatives (SW620) [47]. Using quantitative proteomic analysis, 803 and 787 proteins were identified in the EV4 SW480 and EV6 SW620 lines, respectively. Exosomes obtained from the SW480 cell line were enriched with adhesion-associated proteins (ILK, ITGA1, ICAM1, CTNNB1, PKP2, etc.), while the proteins identified in the exosomes of the SW620 cell line were involved in cancer progression and played a role in the appearance of drug resistance (AARS, CCDC50, PAK4, ANXA1, LCK, LCP1, AK2, HSPA9, etc.) [47].

3.3. Breast Cancer

Studies performed on the MCF10A, MCF7, and MDA-MB-231 breast cancer cell lines have shown that breast cancer exosomes are enriched in certain types of miRNAs (such as miR-21 and miR-1246), the same miRNAs are detected in plasma of PDX mice with breast cancer and their levels are significantly increased in plasma of breast cancer patients [48]. These results support the concept that exosome miRNAs can serve as an important additional diagnostic tool for breast cancer.

Chow et al. found that exosomes from the MDA-MB-231 and MCF7 breast cancer cell lines induced NF-κB activation in macrophages [49].

Italian and Swiss scientists have shown that exosomes secreted by MDA-MB-231 breast cancer cells play a role in tumor metastasis. The authors compared proteomes of isolated exosomes and the whole lysate of breast cancer cells. The proteomic analysis revealed molecules involved in exosome biogenesis and cell cycle regulation (ACTB/G, TBA1C, K1C9, TPM4, FSCN1, TAGL2, and PDC6I), apoptosis regulation (TERA, ANXA5, HSP71, PRDX2, UBIQ, and PDC61) as well as proteins playing an important role in signaling pathways and modulation of the immune system during tumor progression (LG3BP, LAMC1, GBB2, ITA3, ITA6, and B2MG) and focal adhesion (LAMC1, ACTB/G) [50].

3.4. Prostate Cancer

Currently, PSA is widely used in current clinical practice as a PCa marker. However, PSA is an organ-specific marker; in this context its increase not necessarily means tissue malignization. A new trend in the PCa study is the proteomic analysis of prostasomes (prostate specific exosomes) to search for new biomarkers. In the case of PCa, prostasomes appear not only in the prostatic fluid (as it occurs under normal conditions), but also in other peripheral body fluids: blood, urine, and sperm [51].

Semen plasma is an excellent source of biomarkers because it circulates and contacts the reproductive sys-

tem. Proteomic analysis can be used to search biomarkers of infertility and PCa. In a study by Yang et al. exosomes were isolated by ultracentrifugation in a sucrose density gradient, followed by Western blot analysis of samples [52]. The particle size and concentration were determined using the method of analysis of nanoparticle trajectories. The morphology of seminal exosomes was confirmed using transmission electron microscopy. Isolated proteins were determined using HPLC-MS/MS analysis. The semen liquid samples were obtained from 12 healthy donors and randomly divided into two groups for two biological repeats. The analysis resulted in identification of 1474 proteins, including 354 overlapping proteins (from two groups). ALIX and HSP70, annexins and proteins associated with the Ras kinase signaling pathway were used as exosome markers, while intracellular (CNX) proteins used as a negative control. The study, resulted in identification of the following proteins in exosomes isolated from seminal fluid: ACTB, GAPDH, PHGDH, LGALS3BP and SEMG1; as well as biomarkers of male infertility: SEMG1, SEMG2, PIP, FN1, ACPP, KLK3.

Turay et al. compared exosomal proteins obtained from plasma of ethnically different PCa patients. Exosomes were isolated using the commercial ExoQuick kit (SBI, USA) [53]. The list of identified proteins included proteins which were previously described as associated with carcinogenesis and tumor growth: DNA2, PIF1, FHL3, GSTO2, MELK, IRX5, MCM5, MTUS1, etc.

3.5. Ovarian Cancer

Yi et al. have shown that incubation of HUVEC cells with exosomes produced by high-grade ovarian carcinoma cells (SKOV3 and CAOV3) leads to a significant increase in angiogenesis, migration, and changes in cell morphology. Using proteomic analysis, the researchers found 10 exosomal proteins of the CAOV3 cell line associated with angiogenesis, including EBNA1 and MTA1 [54]. Liang et al. studied exosomal proteins of the OVCAR-3 and IGROV1 ovarian cancer cell lines and found their association with carcinogenesis signaling pathways [55]. Proteins overexpressed in ovarian cancer tissue included: EpCAM, PCNA, TUBB, EGFR, APOE, CLDN3. These results have shown that, in addition to common exosomal markers, vesicles contain proteins associated with carcinogenesis and metastasis.

The comparative analysis of serum samples of patients with diagnosed serous papillary adenocarcinoma and primary cell lines obtained from patients with hereditary ovarian cancer (UL-1, UL-2, UL-3, UL-6, UL-B, UL-O) revealed ovarian tumor specific miRNAs associated with tumor tissues (e.g., let-7i, miR-16, miR-21 and miR-214) [56]. In addition, the profiles of exosomal miRNAs coincide with the miRNA profiles of tumor cells; this suggests that exosomal miRNAs reflect the tumor profile and can be used without biopsy.

4. PROTEOME MULTIPLEX PANELS OF MARKERS

The lack of universal markers confirming exosomal identity, as well as tumor-specific exosome markers that would distinguish different types of cancer represents the main problem for the successful application of exosome analysis in the diagnostics of malignant tumors. Simultaneous analysis of the biomarker panel can significantly increase the sensitivity and specificity of diagnostics. Due to a possibility for simultaneous analysis of many proteins in complex biological matrices mass spectrometry is a promising alternative to antibody-based methods.

The capabilities of targeted mass spectrometry were previously used in our laboratory for quantitative analysis of the CD9, CD63, CD82, and HSPA8 markers in exosome samples isolated by differential ultracentrifugation, ultracentrifugation in a sucrose density gradient or using precipitation [21]. According to our data, the sucrose gradient ultracentrifugation appears to be the most suitable for exosome isolation for subsequent mass spectrometry. Shotgun mass spectrometric analysis of the exosomal fraction isolated by this method from the plasma of healthy volunteers resulted in identification of 108 proteins. These included secreted proteins clusterin (CLU), ezrin (EZR), galectin-3 binding protein (LGALS3BP), haptoglobin related protein (HPR), integrin-alpha-IIB (ITGA2B), kallikrein (KLKB1), thrombospondin 1 (THBS1) and vitamin K-dependent protein S (PROS1). These proteins play a role in the signaling pathways associated with various diseases and can be considered as a panel of protein markers.

Most currently performed studies of extracellular vesicles by using proteomic methods as the research tool are aimed at comparative profiling of exosome proteins in cancer patients and healthy volunteers. Despite the fact that the data obtained are of great interest from the viewpoint of searching for diagnostic biomarkers, it is also important to know the molecular features of various forms of cancer within one nosological form.

In order to identify exosome proteins that can discriminate variants of lung adenocarcinoma and colorectal adenocarcinoma, we have performed proteomic profiling of exosomes of two cell lines of lung adenocarcinoma (A549 and NCI-H23) and three cell lines of colorectal adenocarcinoma (HT29, HCT-116 and CaCo-2) (Shushkova et al., in preparation). The studied cell lines differ in the mutational status of the *KRAS*, *BRAF*, *PIK3CA*, *c-MYC* and *p53* genes, which determines different susceptibilities to therapy, especially to targeted drugs. According to the results of high resolution mass spectrometric analysis, 651 pro-

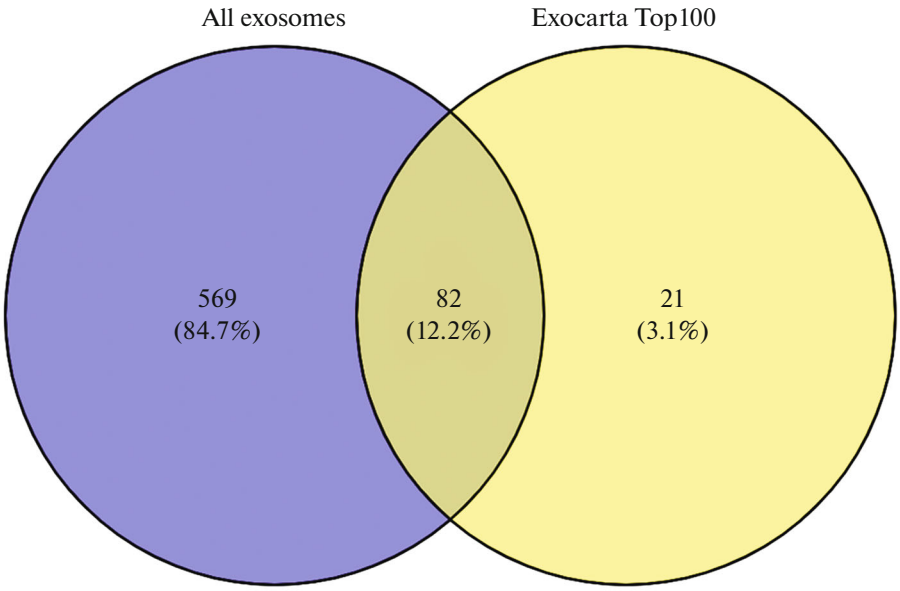

Fig. 3. The Venn diagram for proteins identified in exosome samples of lung adenocarcinoma and colorectal adenocarcinoma (All exosomes) and for the proteins most commonly found in exosomes (based on the ExoCarta database) (Exocarta Top100).

teins were identified by at least two peptides in all exosomal samples. This included 82 proteins from the 103 most frequently found in exosomes (the ExoCarta database materials) (Fig. 3).

Using the relative label-free quantitative analysis, it was possible to determine tissue-specific proteins and proteins specific for each cell line. The list of these molecules included heat shock proteins, integrins, proteins involved in exosome biogenesis, splicing, RNA metabolism, cytoskeleton reorganization and cell adhesion, in the regulation of cell growth, differentiation and apoptosis. Exosome proteins that distinguish selected cell lines can serve as potential predictive and prognostic biomarkers.

CONCLUSIONS

Exosomes preserve the properties of their producing cells and reflect their functions, therefore, the study of the composition of exosomes is a promising area in the search for new minimally invasive biomarkers of malignant tumors. Today, high-performance omics technology is a powerful tool for the molecular study of exosomes. Using genomic and transcriptomic methods it is possible to replicate RNA and DNA molecules, and this helps to "draw the MVs molecular portrait" with the highest sensitivity. At the same time, proteomic methods, primarily mass spectrometry analysis, enable multiplex analysis of proteins that most accurately reflect the tumor phenotype. Most frequently, conditioned medium in which tumor cells have been cultivated, is used as the research object, and promising data at the level of RNA and protein have been obtained for such models. It this context it should be indicated that the validation of tumor markers must be carried out on the biological material from patients, which primarily includes blood and urine, as well as pleural effusion and seminal plasma. These complex biological matrices rather than the primary tumor cells attract much interest as sources of minimally invasive biomarkers. Further research should be directed towards the development of new approaches to the accurate sensitive quantitative analysis of the components of exosomes in complex biological matrices.

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COMPLIANCE WITH ETHICAL STANDARDS

The study did not involve humans or animals as research objects.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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